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ORIGINAL ARTICLE

All-*trans* retinoic acid exhibits anti-proliferative and differentiating activity in Merkel cell carcinoma cells via retinoid pathway modulation

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Abstract

Background: The limited therapies available for treating Merkel cell carcinoma (MCC), a highly aggressive skin neoplasm, still pose clinical challenges, and novel treatments are required. Targeting retinoid signalling with retinoids, such as all-*trans* retinoic acid (ATRA), is a promising and clinically useful antitumor approach. ATRA drives tumour cell differentiation by modulating retinoid signalling, leading to anti-proliferative and pro-apoptotic effects. Although retinoid signalling is dysregulated in MCC, ATRA activity in this tumour is unknown. This study aimed to evaluate the impact of ATRA on the pathological phenotype of MCC cells.

Methods: The effect of ATRA was tested in various Merkel cell polyomavirus-positive and polyomavirus-negative MCC cell lines in terms of cell proliferation, viability, migration and clonogenic abilities. In addition, cell cycle, apoptosis/cell death and the retinoid gene signature were evaluated upon ATRA treatments.

Results: ATRA efficiently impaired MCC cell proliferation and viability in MCC cells. A strong effect in reducing cell migration and clonogenicity was determined in ATRA-treated cells. Moreover, ATRA resulted as strongly effective in arresting cell cycle and inducing apoptosis/cell death in all tested MCC cells. Enrichment analyses indicated that ATRA was effective in modulating the retinoid gene signature in MCC cells to promote cell differentiation pathways, which led to anti-proliferative and pro-apoptotic/cell death effects.

Conclusions: These results underline the potential of retinoid-based therapy for MCC management and might open the way to novel experimental approaches with other retinoids and/or combinatorial treatments.

INTRODUCTION

Merkel cell carcinoma (MCC) is an aggressive skin cancer with an increasing global incidence ranging 0.1–1.6 cases/10^5 subjects/year globally and with a 5-year mortality rate of ~63%.[1,2] Risk factors associated with both MCC aetiologies include fair skin, advanced age and related immunosenescence, anti-MCPyV/–tumour immunological impairment as well as UV radiation exposure.[3] Oncogenic Merkel cell Polyomavirus (MCPyV) is the main causative agent of MCC, as ~80% of cases is MCPyV-positive (MCCP). Remaining MCCs are MCPyV-negative (MCCN).[4]

Effective MCC therapeutic options are limited and mainly include surgery followed by adjuvant radio/chemo therapies.[5–7] The standardized chemotherapy regimen provides the use of platinum-based drugs, such as etoposide, but improvements in terms of survival/therapy response are limited.[8] Immunotherapy based on targeting the receptor–ligand system Programmed cell death protein 1

and programmed death-ligand 1 represent a breakthrough for MCC management.[9] However, its benefit in patientis is frequently hampered by the occurrence of primary and secondary therapy resistance.[10] MCC management still poses a substantial clinical challenge and novel effective therapies are required.[11]

Pre-clinical/clinical evidence underlines the reliability of retinoids, which are active metabolites of vitamin A,[12] as appealing cancer therapeutics.[13,14] The biological activity of retinoids is mediated by retinoid receptors whose signalling transactivate a plethora of genes involved in retinoid-mediated transcriptional responses.[15] Signalling activation regulates cell proliferation and differentiation, cell cycle and apoptosis.[16] Retinoid pathway is dysregulated in various carcinoma types, including MCC.[17] The impairment of several retinoid-associated genes such as SOX2 and ISL1 has been documented in MCC.[18]

The retinoid all-*trans* retinoic acid (ATRA) is a promising and clinically useful antitumor agent. ATRA drives tumour cell differentiation by modulating the retinoid signalling, leading to anti-proliferative/pro-apoptotic effects.[19] Strong antineoplastic and cyto-differentiating activities for ATRA have been demonstrated in myelodysplastic diseases and solid cancers such as lung, breast and prostate carcinomas as well as brain tumours.[20,21] ATRA can suppress the proliferation of primary/metastatic cells in skin neoplasms such as melanoma and inhibit tumour growth in melanoma patients, as documented in clinical trials.[22]

We aimed to evaluate the impact of ATRA treatment on MCC cells. To this purpose, the effect of ATRA on cell proliferation, viability, migration and clonogenic abilities and cell cycle and apoptosis was tested in various MCCP and MCCN cell lines. Gene expression profiles were evaluated in apoptosis/cell death and in retinoid pathway. Our data indicate that ATRA presents anti-proliferative and differentiating activity in MCC cells by modulating the expression of retinoid-associated genes.

MATERIALS AND METHODS

A detailed description of Material and methods is present in File S1.

Cells, compounds and cell proliferation and viability evaluations

MCCN cells MCC13 and MCC26, and MCCP cells PeTa and WaGa and engineered-GFP (eGFP) PeTa-GFP and WaGa-GFP,[23] were cultured in RPMI 1640 medium with 10% FBS (EuroClone, Milan, Italy).[24] Control human fibroblast cells MRC-5 were cultured in DMEM/F-12 medium with 10% FBS (EuroClone, Milan, Italy).[25] ATRA (Sigma-Aldrich, Milan, Italy) was dissolved in 100 mM DMSO.[8,13,14,26] Cell proliferation was tested with WST-1 assay (Merck, Milan, Italy).[14,26] Cell viability was evaluated.[27]

Clonogenic and migration assays

The clonogenic assay was performed to evaluate the ability of cells to produce colonies.[28] Cells were treated/incubated for 72 h at 37°C and 5% CO_2.[29] Cell migration assay was performed to evaluate the ability of cells to close a wound. Results were expressed as mean (±standard deviation [SD]) percentage of cell migration in each check point [(Area t0 hours—Area tx hours)/(Area t0 hours)] × 100.[29] Measurements were performed with ImageJ software.

Cell cycle evaluation

Cells were harvested and centrifuged at 500 × *g* for 5 min at 4°C. The medium was discarded, and cells suspended in PBS, centrifuged at 500 × *g* for 5 min and transferred to ice. Cells were fixed overnight with ice-cold 70% ethanol in distilled water. The next day, cells were washed, centrifuged and stained with the Tali® Cell Cycle Solution for 30 min at room temperature. Data were acquired with the Tali® ImageBased Cytometer according to the manufacturer's protocol.

Gene expression analysis

The RT^2 Profiler PCR Array Human Apoptosis and RT^2 Human Retinoic Acid Signaling PCR array were used to measure the expression levels of 84 apoptosis-associated genes and 84 retinoid-associated genes, respectively. Kits/arrays procedures were performed according to the manufacturer's protocols (Qiagen, Milan, Italy and Bio-Rad, Milan, Italy).[18]

Western blot

Cells were collected and lysed in RIPA Lysis and Extraction Buffer (Thermo, Milan, Italy). Protein lysate concentrations were quantified using the Pierce™ BCA Protein Assay Kit (Thermo, Milan, Italy).[30] Proteins were separated in Mini-PROTEAN® TGX Stain-Free™ Protein Gels and transferred to membranes via Trans-Blot® Turbo™ Transfer System (Bio-Rad, Milan, Italy). The list of antibodies employed is included in File S1.

Annexin-V/PI assay

Cells were gently harvested, washed with buffers and incubated for 20 min at room temperature with annexin V-Cy3 solution (Biovision, Milpitas, CA, USA). Cells were washed with buffers and incubated for 3 min at room temperature with PI solution. The green (Annexin-V) and red (PI) fluorescence signals were quantified under all conditions on a Tali image-based cytometer (Thermo, Milan, Italy).

Statistical analysis

Analyses were performed with MedCalc v.16.2.1 and GraphPad Prism v.8.0.[31] $p < 0.05$ was considered statistically significant.[32] Hierarchical clustering and principal component analysis (PCA) were performed using ClustVis. Gene ontology (GO)/Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were performed to categorize genes. Protein–protein interaction (PPI) network was constructed using STRING.[33] Three PPI hub genes/cell line were identified via cytoHubba.[34]

RESULTS

ATRA inhibits Merkel cell carcinoma cell proliferation and viability

Cell proliferation was tested in MCCN cells MCC13 and MCC26 and MCCP cells PeTa and WaGa for 24, 48 and 72 h (Figure 1b). ATRA was added at different concentrations to determine IC_{50s}. MCC13, PeTa and WaGa cells proved to be ATRA sensitive, with IC_{50s} ranging from 75 μM for PeTa to 100 μM for MCC13 and WaGa at 72 h. ATRA slightly affected MCC26 cell proliferation and did not affect that of MRC-5. IC_{50} values were employed for subsequent in vitro experiments, while the highest value determined in MCC cells, that is, 100 μM, was chosen for MCC26 and MRC-5.

Cell viability was tested on MCCP suspension PeTa-GFP and WaGa-GFP cells (Figure 1c). The mean rate of fluorescent cells at 48 and 72 h was significantly lower in ATRA-treated PeTa-GFP and WaGa-GFP cells compared to corresponding control conditions ($p < 0.05$). The mean rate of fluorescent cells at 48 and 72 h was significantly lower in etoposide-treated PeTa-GFP and WaGa-GFP compared to control conditions ($p < 0.05$).

ATRA exhibits anti-clonogenic and anti-migratory effects in Merkel cell carcinoma cells

The effect of ATRA on cell clonogenicity and migration was evaluated in MCCN adherent MCC13 and MCC26 cells and in the control cell line MRC-5 (Figure 1).

Cell clonogenicity was tested after 72 h of treatments (Figure 1d). A significant reduction in clonogenicity was determined in ATRA-treated MCC13 and MCC26 cells compared to corresponding control conditions ($p < 0.05$). A slight, but non-significant, effect was observed in ATRA-treated MRC-5 cells compared to untreated cells ($p > 0.05$). Cell migration was evaluated at 24, 48 and 72 h of treatment (Figure 1e). A strong anti-migratory effect was found in ATRA-treated MCC13 and MCC26, whose mean percentage of migrating cells at both 48 and 72 h was significantly lower compared to corresponding control conditions (Figure 1e, $p < 0.0001$). The migration of MRC-5 cells was not influenced by either ATRA or etoposide at 24, 48 and 72 h ($p > 0.05$).

ATRA induced cell cycle arrest in Merkel cell carcinoma cells

Cell cycle was evaluated after 72 h of treatments. In MCC13 and MCC26, ATRA increased the number of cells in G1/G0 phase (Figure 2). Similarly, the fraction of WaGa and Peta cells in G1/G0 phase increased in ATRA condition. A high percentage of G1/G0 phase cells was noted in MCC13 and WaGa cells. A high fraction of cancer cells in G1/G0 phase was detectable in etoposide-treated cells, both for MCCN and MCCP cells. ATRA did not lead to an increase in the number of control cells MRC-5 in G1/G0 phase.

ATRA modulates the apoptotic gene signature of Merkel cell carcinoma cells

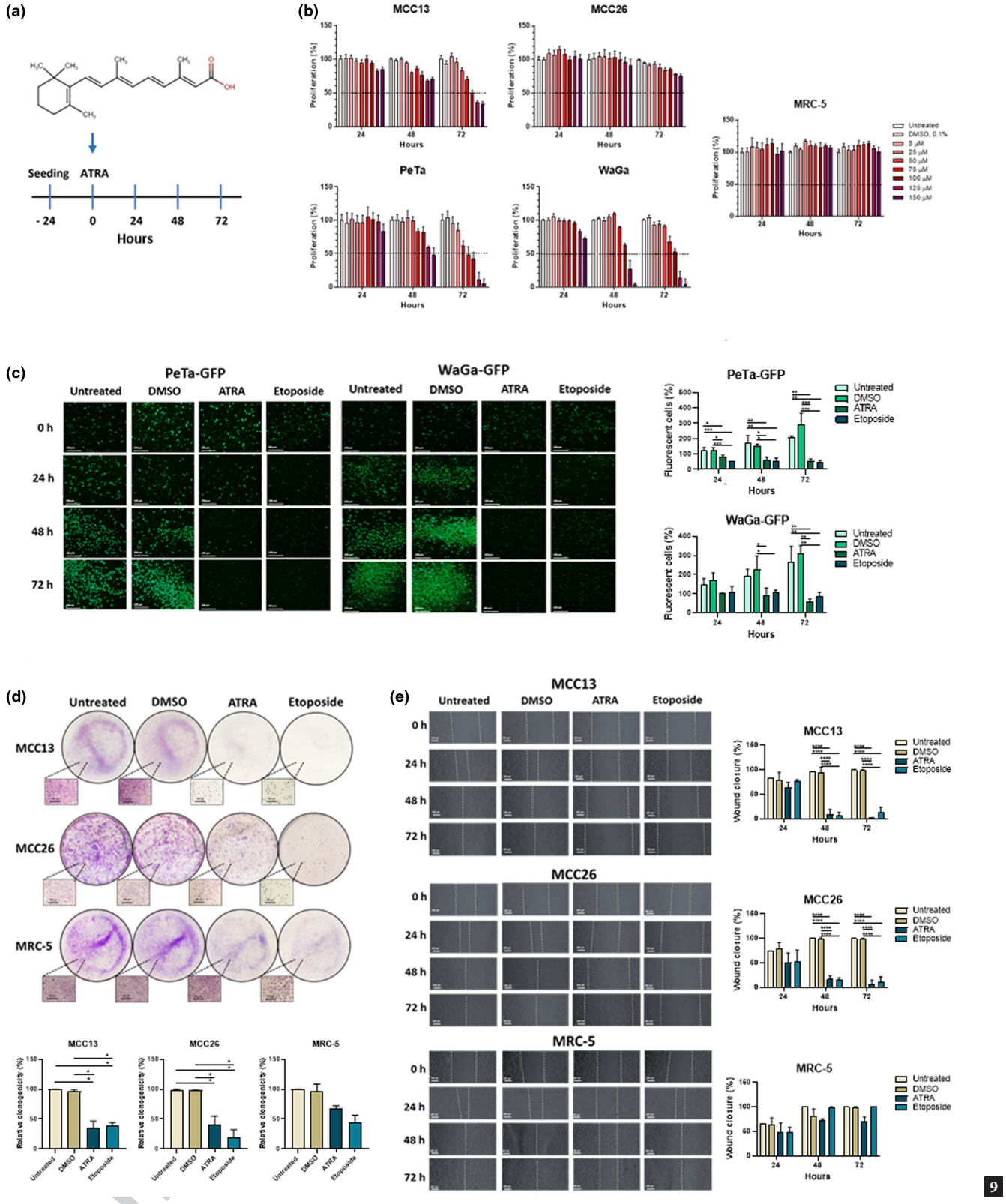
The apoptotic gene signature was interrogated in ATRA-treated versus untreated MCC cells via qPCR array after 72 h of treatments. Two clustered heatmaps illustrating the apoptotic gene signature across MCC cell lines are depicted in Figure 3. A remarkably positive modulation of pro-apoptotic genes was observable in all ATRA-treated MCC cells (Figure 3a–d). GO enrichment analysis for biological processes and KEGG analysis indicated that apoptotic-related processes were the most enriched biological processes in MCC cells (Figure 3b,d).

ATRA prompts apoptosis/cell death in Merkel cell carcinoma cells

The effect of ATRA treatment in MCC cells was evaluated using Annexin-V/PI assays after 72 h of treatments (Figure 4). The fraction of annexin-V-positive MCC13, MCC26, PeTa and WaGa cells treated with ATRA was significantly higher compared to corresponding control conditions ($p < 0.05$), while ATRA reduced apoptosis in MRC-5 cells compared to corresponding control conditions ($p < 0.05$, Figure 4a). ATRA-treated MCC13, MCC26, PeTa and WaGa cells exhibited a significant increase in necrotic cells, compared to corresponding control conditions ($p < 0.05$, Figure 4b). ATRA-treated MRC-5 cells did not show any difference in terms of necrotic cells compared to untreated cells ($p > 0.05$).

ATRA induces the expression of pro-apoptotic proteins in Merkel cell carcinoma cells

The expression of anti-apoptotic proteins full-length PARP-1, BCL-XL and BCL-2 and pro-apoptotic proteins cleaved PARP-1 and cleaved caspase-7 was evaluated by western



blot in MCC and control cells after 72 h of treatments (Figure 5a–e).

All ATRA-treated MCC cells exhibited significantly higher levels of cleaved PARP-1 and cleaved caspase-7 (excluded WaGa) compared to corresponding control conditions, where the signal was undetectable ($p < 0.01$). A concomitantly significant PARP-1 full-length level decrease was noted in all ATRA-treated MCC cells in comparison with corresponding control conditions ($p < 0.05$). Moreover, BCL-XL levels were found to be strongly reduced in ATRA-treated MCC26 cells compared to corresponding control conditions ($p < 0.05$), while protein expression did not change

FIGURE 1 All-*trans* retinoic acid (ATRA) treatment in Merkel cell carcinoma (MCC) cells and fibroblast control cell line. (a) Chemical structure of all-*trans* retinoic acid (ATRA) and treatment schedule. (b) Effect of ATRA on cell proliferation in MCC cells and fibroblast control cells. (c) Effect of ATRA on cell viability of GFP-engineered PeTa and WaGa cell lines, that is, PeTa-GFP and WaGa-GFP. Cell viability was evaluated by fluorescence in PeTa-GFP and WaGa-GFP cells at 24 and 72 h after treatment. Graphical data represent cell viability after treatment over untreated control values. Images were captured at 0 h directly after cell seeding. Cell viability is presented as the percentage (%) of fluorescence measured at 24 and 72 h after treatment compared to control values. Untreated cells were used as controls. (d) Colony formation assay performed on MCC13, MCC26 and MRC-5 cells. Cell colonies were stained with 0.5% crystal violet dye. Graphical data represent the level (%) of colony formation after exposure to ATRA compared to untreated cells. Values are presented as mean ± SD, and statistical significances are indicated as $^{*}p < 0.05$. (e) Cell migration assay in MCC13, MCC26 and MRC-5 cells. Images were captured at $t = 0$ h directly after scratching the cell layer, $t = 24$ and $t = 72$ h to evaluate scratch (wound) closure indicative of cellular migration. The cell migration level is presented as the percentage of wound closure observed at 24 and 72 h after treatment compared to control values. Controls indicate untreated cells. All panels $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$.

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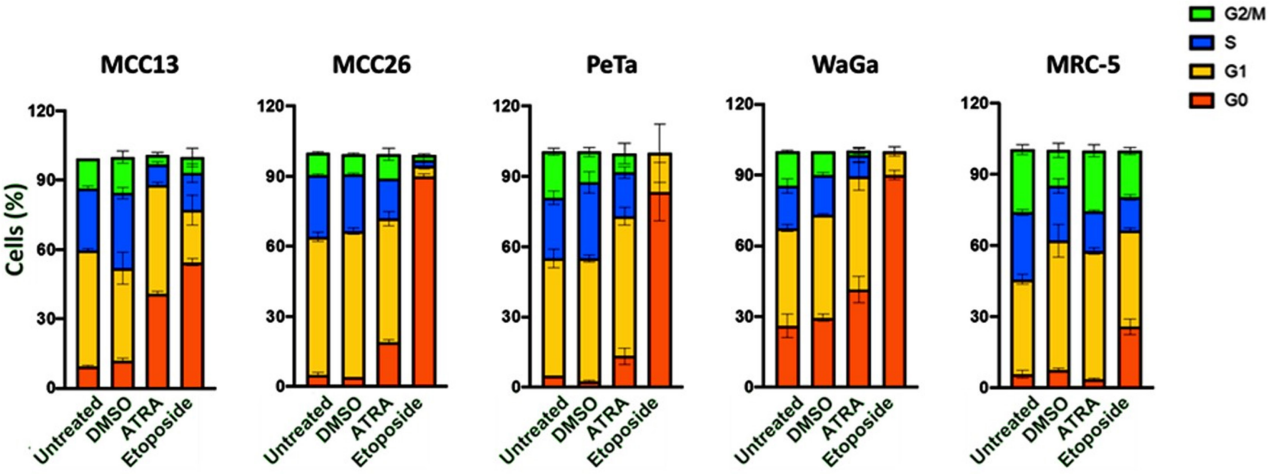

FIGURE 2 Cell cycle evaluation in Merkel cell carcinoma (MCC) cells MCC13, MCC26, PeTa, WaGa and in the fibroblast control cell line MRC-5. Analyses were performed after 72 h of treatment with all-*trans* retinoic acid (ATRA), etoposide and DMSO. Graph bars represented the % of cell population in different phases of the cell cycle. ATRA induced G0/G1 cell cycle arrest in MCC13, MCC26, PeTa, WaGa cells, without perturbing MRC-5 cells. All bars represent means ± SD.

in MCC13, PeTa and WaGa cells across all experimental conditions ($p > 0.05$, Figure 5a–d). PeTa and WaGa cells exhibited similar BCL-2 levels across all experimental conditions ($p > 0.05$), while the same protein was undetectable both in MCC13 and MCC26, in any conditions (Figure 5a,b), as reported.[35]

Concerning MRC-5 cells, similar BCL-XL and cleaved caspase-7 levels were determined among all experimental conditions upon ATRA treatments ($p > 0.05$). This effect was paralleled with a slight but significant increase in BCL-2 levels in ATRA-treated cells compared to control conditions ($p < 0.05$, Figure 5e). Slight, but significantly higher, cleaved PARP-1 levels were determined in ATRA-treated cells, compared to control conditions (Figure 5e, $p < 0.05$).

Retinoid gene expression, protein–protein interaction and hub genes identification

The molecular response to ATRA was interrogated by evaluating the expression of 84 retinoid genes in MCC cells, using qPCR array, after 72 h of treatments (Figure 6).

A PPI network was constructed with modulated retinoid genes to identify gene interactions (Figure 6). In MCC13 cells, 22 nodes (genes) and 41 edges (interactions) were identified, while MCC26 cells exhibited 21 nodes and 32 edges; a total of 26 nodes and 73 edges were identified in PeTa, while WaGa cells exhibited 31 nodes and 100 edges ($p < 1.0 \times 10^{-16}$). The average node degree, clustering coefficient and expected number of edges ranged 21–31, 3.05–6.45 and 4–9, respectively. GATA4, SHH and PAX6 were upregulated hub genes in MCC13, while MCC26 exhibited SOX2 as a downregulated hub gene and SHH and KLF4 as upregulated hub genes. GATA4, FGF8 and SHH were upregulated hub genes in PeTa. WaGa exhibited SOX2 and ALDH1A3 as downregulated and upregulated hub genes, respectively, while SHH resulted as an upregulated hub gene in two out of four algorithms.

ATRA modulates the retinoid pathway in Merkel cell carcinoma cells

Two clustered heatmaps illustrating retinoid gene signatures indicated that genes were modulated in all ATRA-treated MCC cells (Figure 7). Hierarchical stratification according to the data matrix indicated that while MCCN cells were categorized in unique branches

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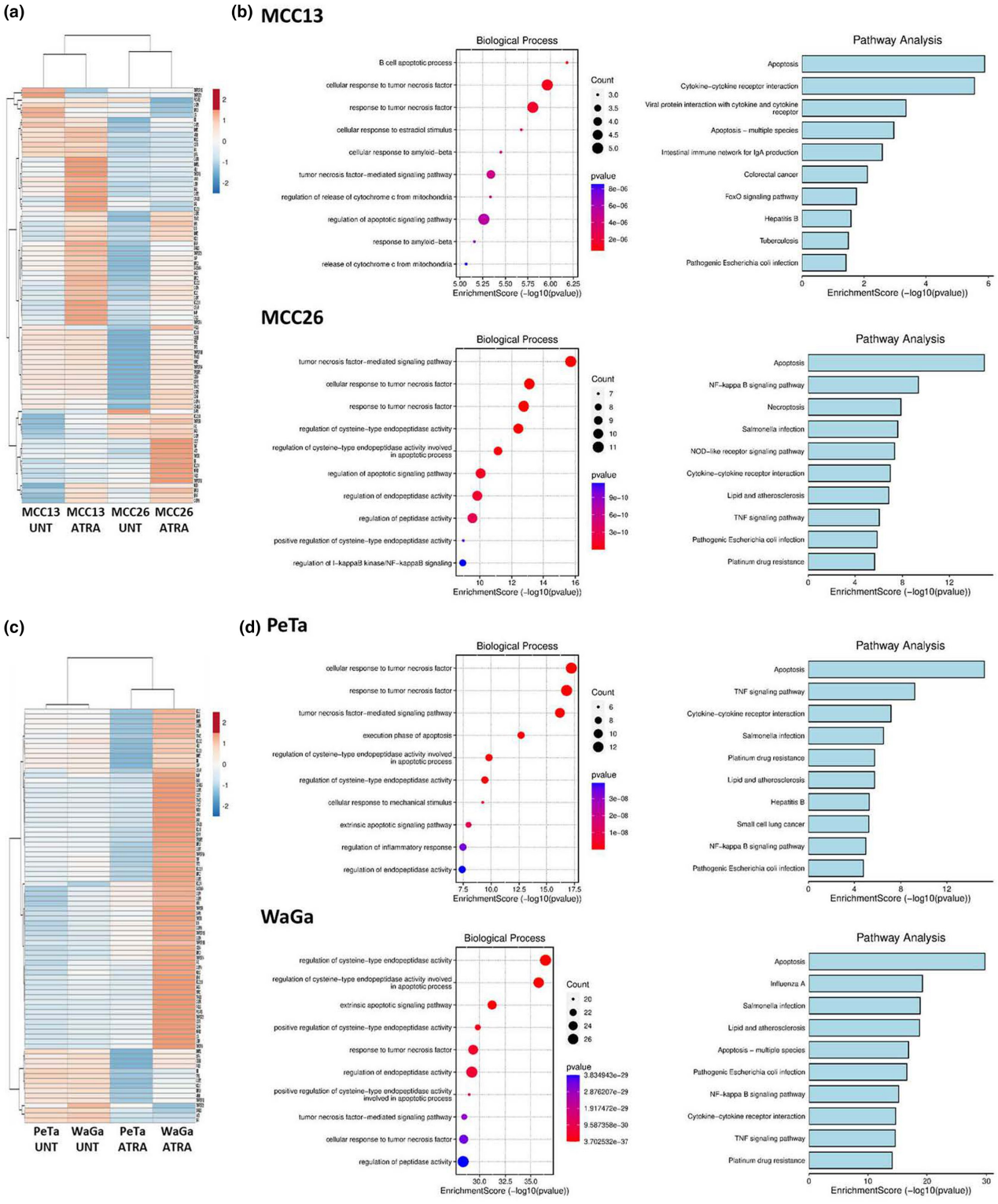

FIGURE 3 Clustered heatmap of 84 pro-/anti-apoptotic genes in all-*trans* retinoic acid (ATRA) treated Merkel cell carcinoma (MCC) cells MCC13, MCC26, PeTa and WaGa compared to untreated cells and functional enrichment analyses of significantly modulated genes. Experiments were performed by qPCR array, after 72 h of treatments. (a, c) Gene symbols are depicted on the y-axis, on the right. Heatmap data matrix of the pro-/anti-apoptotic gene signature visualizes the values in cells using a red-to-blue colour gradient which gives an overview from the highest to the lowest expression values in the matrix. (b, d) Bubble plots represent the signalling pathways of the entire set of significantly modulated apoptotic genes for each MCC cell line resulting in the comparison between ATRA-treated and corresponding untreated cells using Gene ontology (GO) database. Bar plot depicts the signalling pathways of the entire set of significantly modulated apoptotic genes for each MCC cell line resulting in the comparison between ATRA-treated and corresponding untreated cells using Kyoto Encyclopedia of Genes (KEGG) database. Enrichment analyses show GO for biological process terms and KEGG pathways. Heatmaps were performed using Clustvis software, while GO and KEGG enrichment were plotted by https://www.bioinformatics.com.cn/en.

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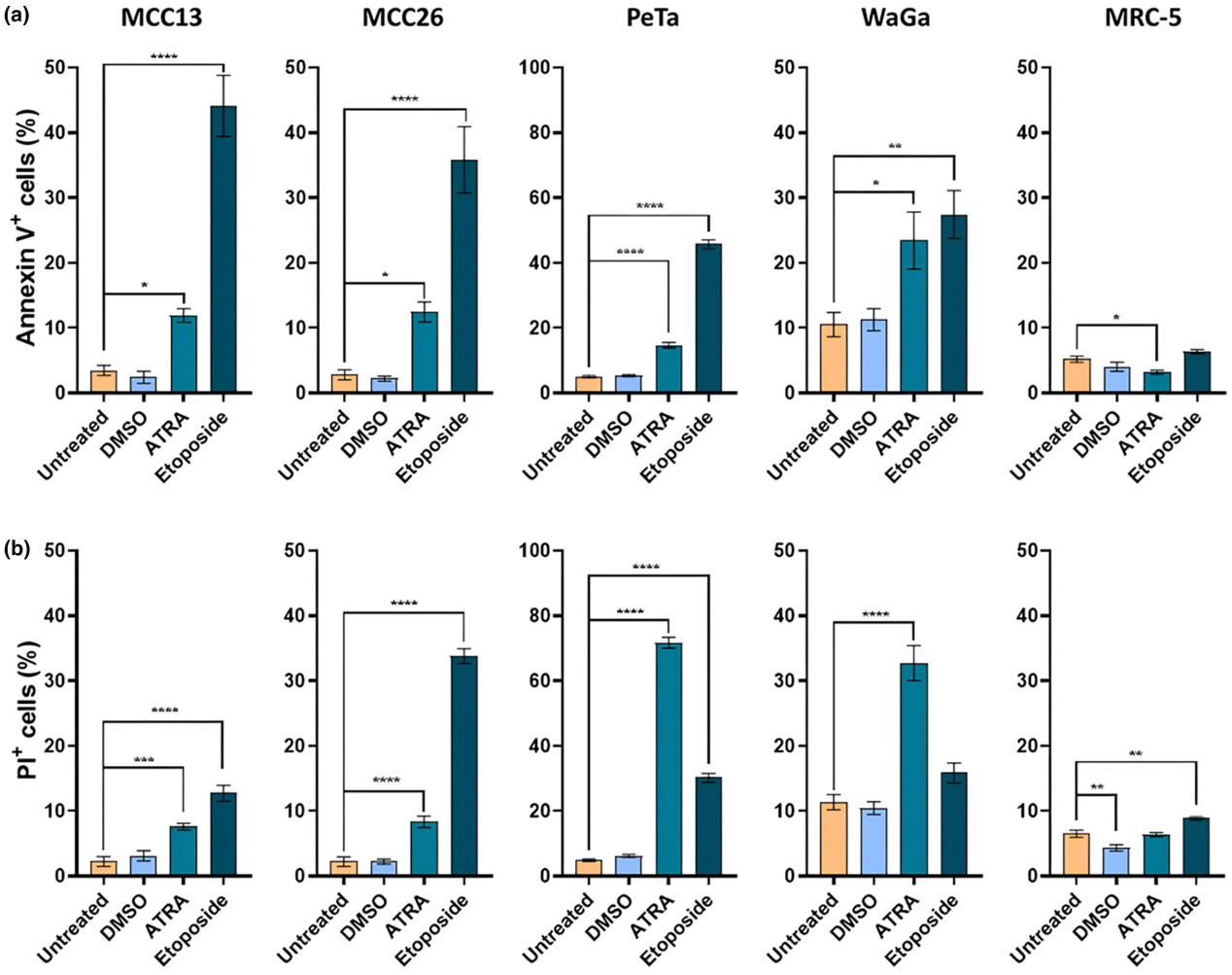

FIGURE 4 Apoptosis and necrosis evaluation via Annexin V and PI assays, respectively, in Merkel cell carcinoma (MCC) cells MCC13, MCC26, PeTa, WaGa and in fibroblast control cell line MRC-5. (a, b) Bar graphs represent the % of apoptotic and necrotic cells after 72 h of treatments in MCC and MRC-5 cells. Apoptotic cells were considered as Annexin V-positive/PI-negative and-positive cells while necrotic cells were considered as Annexin V-negative/PI-positive cells. All bars represent means ± SD. All panels: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$.

independently from ATRA (Figure 7a), ATRA-treated MCCP cells were hierarchically separated from untreated MCCP cells (Figure 7c). Two-dimensional PCA plots indicated that MCCN cells were separable according to cell type, as both ATRA-treated/untreated MCC26 cells were grouped in specific branches and separated from corresponding MCC13 cells. PCA plots demonstrated a separation of ATRA-treated MCCP cells from untreated MCCP cells (Figure 7a,c).

Upon ATRA treatment, embryonic organ morphogenesis/development were the most enriched GO biological process pathways in MCCN. MCCP cells exhibited ureteric bud/mesonephric epithelium and tubule/mesonephros/renal system development as the most enriched biological processes pathways. WaGa cells exhibited regionalization, pattern specification processes and lung epithelium/respiratory system development as the most significantly enriched biological process pathways in upregulated genes. KEGG analysis illustrated that signalling pathways regulating pluripotency of stem cells, basal cell/gastric cancers, retinol metabolism and the PPAR signalling pathway as the most enriched pathways in MCC cell (Figure 7b,d).

DISCUSSION

With the hypothesis that ATRA may present anti-proliferative and differentiating abilities in MCC, we aimed to evaluate the impact of this compound on MCC cells.[36] Dose–response experiments indicated variable IC_{50s} across MCC13, WaGa and Peta cell lines.[13] Moreover, cell proliferation was modestly affected with high concentrations in MCC26 and unperturbed in fibroblast control cells. Consistently, ATRA reduced the viability of MCCP cells. These data indicate that ATRA inhibits MCC cell proliferation and underline the heterogeneity of MCC in the response to ATRA.[18,37] Cell clonogenicity and migration resulted as inhibited in ATRA-treated MCCN cells, while no effect was detected in control cells. Given the demonstrated modulatory activity of ATRA on multiple pathways and its powerful anti-proliferative

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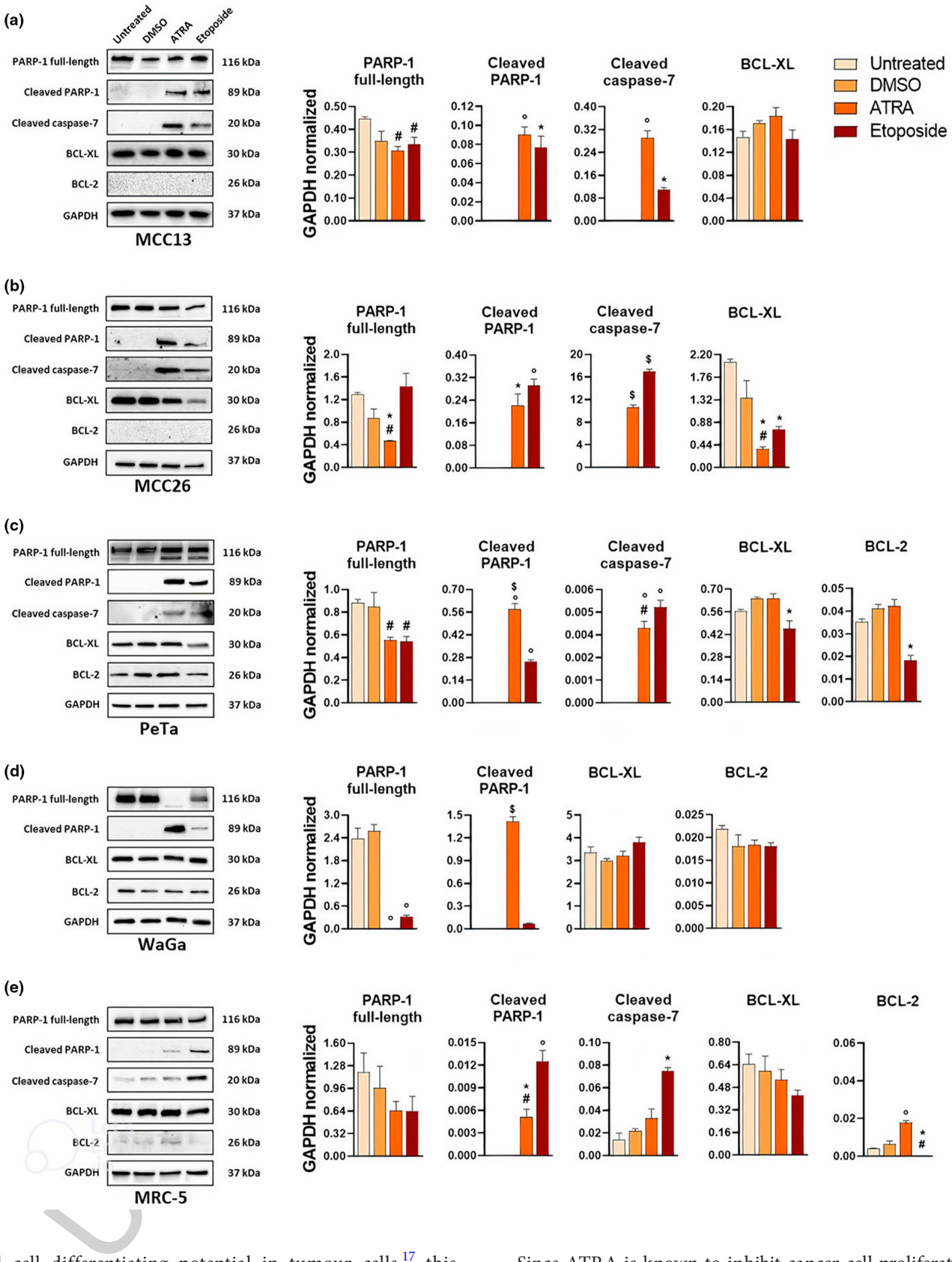


and cell differentiating potential in tumour cells,[17] this retinoid is employed for cancer therapy.[12] Indeed, ATRA has been administered in patients and resulted as useful in managing myelodysplastic diseases,[14,21] solid tumours[20,38] and melanoma.[22]

Since ATRA is known to inhibit cancer cell proliferation by regulating cell cycle,[39] we assessed the cell cycle of MCC cells upon ATRA treatment. ATRA induced G0/G1 cell cycle arrest in MCC cells, without perturbing control cells. ATRA is known to modulate either the RAR/RXR or MAPK pathway,

FIGURE 5 Pro-/anti-apoptotic protein investigation in Merkel cell carcinoma (MCC) cells MCC13, MCC26, PeTa, WaGa and in the fibroblast control cell line MRC-5 after 3-days of all-*trans* retinoic acid (ATRA) treatment by western blot analysis. (a–e) Apoptosis was evaluated by western blot (WB) analysis after 72 h of treatments using selective antibodies against PARP-1 full length, cleaved PARP-1, cleaved caspase-7, BCL-XL and BCL-2. Apoptosis was evaluated by the densitometry quantification of protein levels of PARP-1 full-length (116 kDa), cleaved PARP-1 (89 kDa), cleaved caspase-7 (20 kDa), BCL-XL (30 kDa) and BCL-2 (26 kDa) and normalized to GAPDH (37 kDa). Results are shown as mean ± standard error of mean (SEM). All panels: untreated, unt; etoposide, eto. (a) PARP-1 full-length: $^{\#}p < 0.05$, ATRA versus unt, eto versus unt. Cleaved PARP-1: $^{\circ}p < 0.001$, ATRA versus unt and DMSO; $^{*}p < 0.01$, eto versus unt and DMSO. Cleaved caspase-7: $^{\circ}p < 0.001$, ATRA versus unt, DMSO and eto; $^{*}p < 0.01$, eto versus unt and DMSO. (b) PARP-1 full-length: $^{\#}p < 0.05$, ATRA versus unt; $^{*}p < 0.01$, ATRA versus eto. Cleaved PARP-1: $^{*}p < 0.01$, ATRA versus unt and DMSO; $^{\circ}p < 0.001$, eto versus unt and DMSO. Cleaved caspase-7: $^{\$}p < 0.0001$, ATRA versus unt and DMSO, ATRA versus eto, eto versus unt and DMSO. BCL-XL: $^{\#}p < 0.05$, ATRA versus DMSO; $^{*}p < 0.01$, ATRA versus unt, eto versus unt. (c) PARP-1 full-length: $^{\#}p < 0.05$, ATRA versus unt and DMSO, eto versus unt and DMSO. Cleaved PARP-1: $^{\$}p < 0.0001$, ATRA versus unt and DMSO; $^{\circ}p < 0.001$, eto versus unt and DMSO, ATRA versus eto. Cleaved caspase-7: $^{\circ}p < 0.001$, ATRA versus unt and DMSO, eto versus unt and DMSO; $^{\#}p < 0.05$, ATRA versus eto. BCL-XL: $^{*}p < 0.01$, eto versus ATRA and DMSO. BCL-2: $^{*}p < 0.01$, eto versus ATRA, unt and DMSO. (d) PARP-1 full-length: $^{\circ}p < 0.001$, ATRA versus untreated and DMSO, eto versus unt and DMSO. Cleaved PARP-1: $^{\$}p < 0.0001$, ATRA versus unt, DMSO and eto. (e) Cleaved PARP-1: $^{\#}p < 0.05$, ATRA versus unt and DMSO; $^{*}p < 0.01$, ATRA versus eto; $^{\circ}p < 0.001$, eto versus unt and DMSO. Cleaved caspase-7: $^{*}p < 0.01$, eto versus unt, DMSO and ATRA; BCL-2: $^{\#}p < 0.05$, eto versus unt; $^{*}p < 0.01$, eto versus DMSO; $^{\circ}p < 0.001$, ATRA versus unt, DMSO and eto.

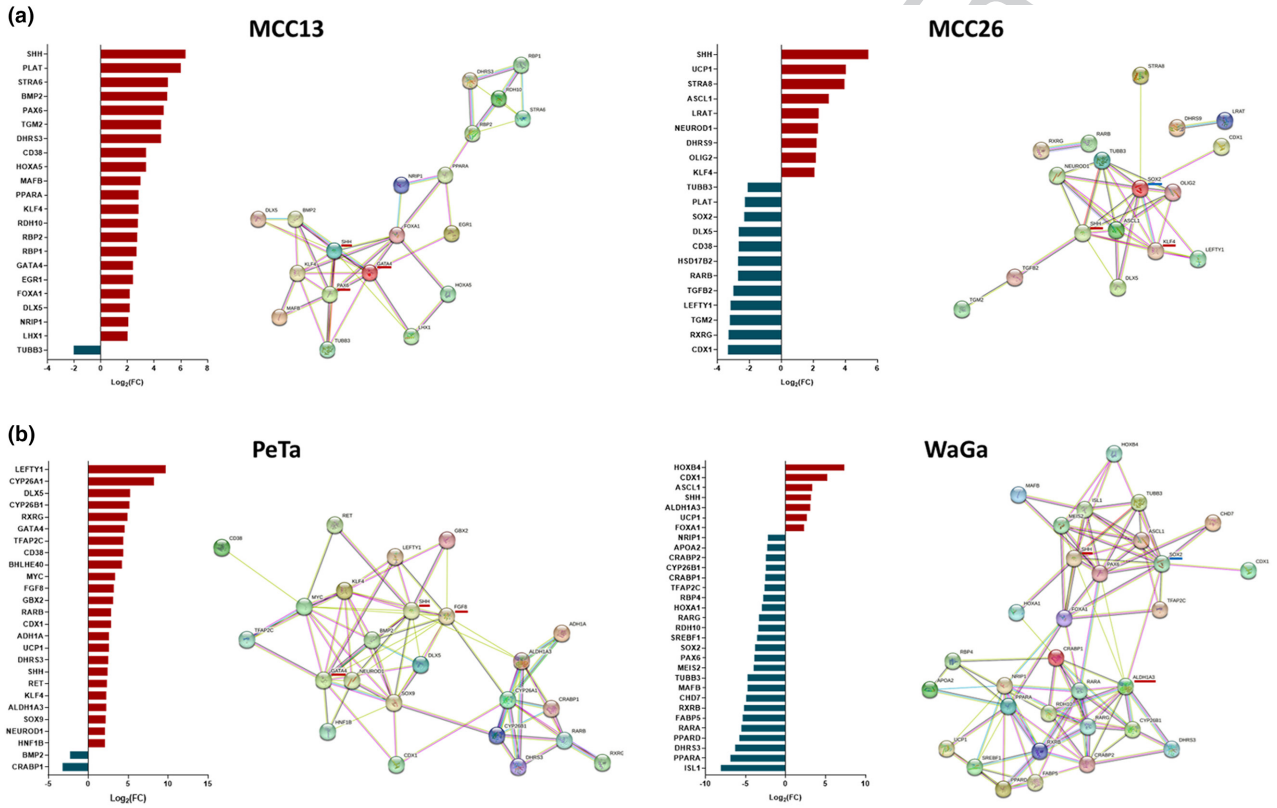
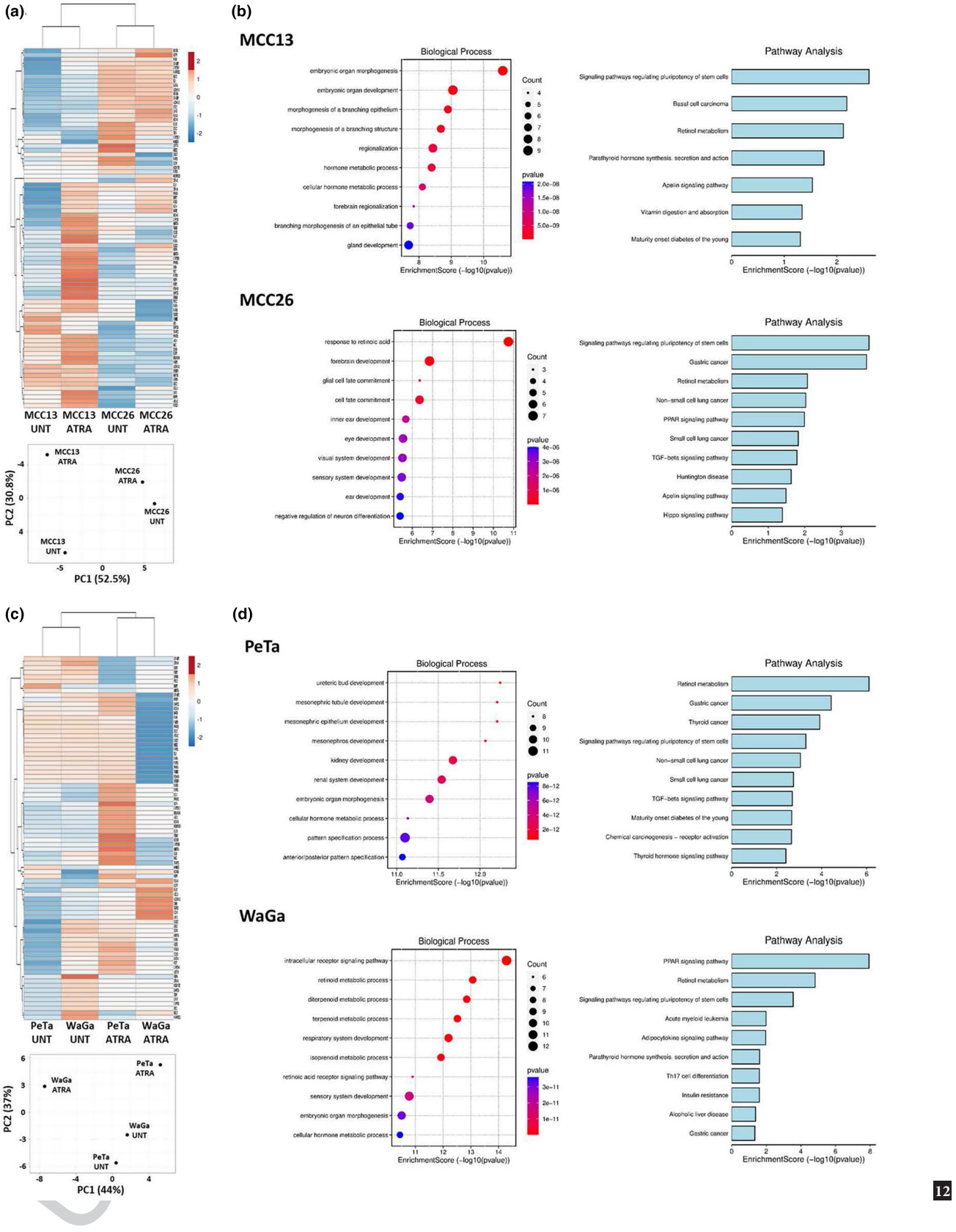


FIGURE 6 Significantly modulated retinoid genes between all-*trans* retinoic acid (ATRA) treated Merkel cell carcinoma (MCC) cells MCC13, MCC26, PeTa, WaGa and corresponding untreated cells. (a) Graphical representation of significantly modulated retinoid genes in ATRA-treated MCC13 and MCC26 versus corresponding untreated cells. A protein–protein interaction (PPI) network was constructed using the STRING database with the entire set of 22 and 21 modulated retinoid genes between ATRA-treated and untreated MCC13, MCC26, respectively. (b) Graphical representation of modulated retinoid genes in ATRA-treated PeTa and WaGa versus untreated cells. A PPI network was constructed with the entire set of 26 and 31 retinoic-associated modulated retinoid genes between ATRA-treated and untreated PeTa, WaGa, respectively. Three hub genes were identified using Cytoscape. Hub upregulated genes between MCC ATRA-treated and untreated MCC cell lines are underlined in red, while hub downregulated genes are underlined in blue. All panels: red indicates upregulated (log2 FC > 2) and blue downregulated genes (log2 FC < –2). Network nodes correspond to genes, while edges represent associations.

leading to cell differentiation and G0/G1 cell cycle arrest.[40,41] Our findings support previous studies reporting that ATRA regulates cell cycle to inhibit tumour cell proliferation, possibly through a differentiation-induced cell cycle arrest process.[41]

Herein, a strong modulation of apoptotic genes was determined in ATRA-treated MCC cells, while enrichment analyses indicated that modulated genes were predominantly involved in apoptosis/necrosis-related pathways. Consistently, apoptotic protein evaluation indicated PARP-1 and caspase 7 activation, paralleled with a reduction in BCL-XL and BCL-2 in the vast majority of ATRA-treated MCC cells.[42] Our data may cumulatively suggest that ATRA



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can drive MCC cells to cell death by eliciting the expression of apoptosis/necrosis-related pathways and activating pro-apoptotic proteins.[42] The significant increase in both apoptotic and death MCC cells determined by annexin-V/PI staining, corroborated this hypothesis. Apoptosis was also evaluated in control cells, where a weak increase was determined for cleaved PARP-1, only. These data underline the reliability of ATRA as an anti-proliferative and pro-apoptotic

FIGURE 7 Clustered heatmap and principal component analysis (PCA) of 84 retinoid-associated genes in all-*trans* retinoic acid (ATRA) treated Merkel cell carcinoma (MCC) cells MCC13, MCC26, PeTa and WaGa compared to corresponding untreated cells and functional enrichment analyses of significantly modulated retinoid genes. (a, c) Gene symbols are depicted on the y-axis, on the right. Heatmap data matrix of the retinoid gene signature visualizes the values in cells using a red-to-blue colour gradient that gives an overview from the highest to the lowest expression values in the matrix. PCA analysis of retinoid genes in ATRA-treated and untreated MCC cells. The two-dimensional PCA plots comprises x and y axes which depict PC1 and PC2 for (a) MCC13 and MCC26 and (c) PeTa and WaGa. (b, d) Bubble plots show pathways of the whole set of significantly modulated retinoid genes for each MCC cell line resulting in the comparison between ATRA-treated and untreated cells using Gene ontology (GO) database. Bar plots show the signalling pathways of the entire set of significantly modulated retinoid genes for each MCC cell line resulting in the comparison between ATRA-treated and untreated cells using the Kyoto Encyclopedia of Genes (KEGG) database. Enrichment analyses represent GO for biological process terms and KEGG pathways. Heatmaps and PCA plots were performed using Clustvis software, while GO and KEGG enrichment were plotted by https://www.bioinformatics.com.cn/en.

agent in MCC. ATRA can drive MCC cells to programmed death through activating apoptotic signals.[22]

Retinoids such as ATRA are powerful cell differentiation inducers.[17] Signalling activation stimulates tumour cell differentiation, reverting, in turn, the malignant phenotype.[17,43,44] The potential of ATRA in inducing anti-proliferative effects in MCC cells through a differentiating mechanism was next explored in terms of retinoid gene signature evaluation. GO analysis revealed that genes modulated by ATRA were mostly enriched in response to retinoic acid-/differentiation-related pathways, such as cell fate commitment, organ, and epithelial morphogenesis/development and regionalization. These data might suggest that ATRA can drive MCC cell differentiation by modulating differentiation pathways, confirming the differentiating potential of this compound in cancer cells.[17,43,44] This hypothesis was confirmed by the PPI network and the through the identification of the hub genes modulated by ATRA. Indeed, the majority of identified hub genes, including SHH, GATA4, KLF4 and PAX6, encode for transcription factors involved in development/differentiation. In particular, SHH controls stem cell/neural development/patterning and Merkel cell specification,[18,45] while GATA4 and KLF4 promote endoderm and epithelial differentiation,[46,47] respectively. PAX6 controls the nervous system development/function of eyes and pancreas and is required for Merkel cell development.[48] SOX2, identified herein as downregulated hub gene, controls embryonic neural crest stem cell maintenance and Merkel cell lineage. Notably, SOX2 silencing in MCC cells can convert the cancer cell phenotype to a differentiated neuron-like phenotype.[49] ATRA might therefore elicit differentiation in MCC cells reverting, in turn, their malignant phenotype, through the downregulation of SOX2 as a contributing mechanism. The remaining two hub genes, FGF8 and ALDH1A3, play a role in organogenesis and in retinoic acid production,[50,51] respectively. Our novel findings demonstrate that ATRA can modulate retinoid hub genes and pathways to promote MCC cell differentiation, leading to anti-proliferative and pro-apoptotic/cell death effects.

Heatmap analysis revealed that retinoid genes were modulated by ATRA in MCC cells. However, MCCP cells were hierarchically categorized according to experimental conditions, while MCCN cells were grouped independently from ATRA. PCA analysis confirmed these findings. Also, an increased rate of significantly modulated genes was observed in MCCP, as opposed to MCCN. Thus, the molecular response to ATRA appeared to be more pronounced in MCCP, supporting previously reported differences in the retinoid gene signature between the two MCC subsets.[18]

Pluripotency of stem cells, basal cell/gastric cancers and PPAR signalling were determined herein as the most enriched KEGG pathways. This indicate that ATRA-treated cells might (i) present more common features with other malignancies than untreated cells; (ii) express stem cell pluripotency/energy homeostasis and metabolic markers. Hence, ATRA might be implicated in the regulation of energy metabolism plasticity in MCC cells which is known to control the balance between stem cell pluripotency and lineage specification.[52]

Identifying ATRA concentrations that are higher (>10 μM) than those commonly determined in vitro for a typical antitumor agent may be a study limitation. However, ATRA has previously been tested in various in vitro tumour models with concentrations similar to those obtained herein.[19,53] Another limitation is the lack of in vivo validation of our data. ATRA is an approved compound which is currently administered in myelodysplastic patients, resulting less toxic than conventional therapy.[38]

In conclusion, ATRA modulated the retinoid pathway in MCC cells to promote cell differentiation, leading to anti-proliferative and pro-apoptotic/cell death effects. Our novel data support previously reported findings indicating the strong anti-proliferative and cyto-differentiating potential of ATRA in cancer cells.

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CONFLICT OF INTEREST STATEMENT

The authors of the present manuscript have no conflicts of interest to declare.

DATA AVAILABILITY STATEMENT

All data generated or analysed during this study are included in this article and its Supplementary Material files. Further enquiries can be directed to the corresponding author.

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